10/4/99

10/4 Characterization of Clone 7-Derived hSTNFRI
- Binding to TNF (Inhibition by H398 α-STNFRI Ab)

H398: mouse (no Ab) α-hTNFRI but also recognizes the soluble fragment Cat # AHR3011 Lot # 10010-015

We want to further characterize the biological activity of Clone 7-derived hSTNFRI. Namely, we want to determine if a neutralizing Ab to STNFRI, H398 (BioSource), can inhibit the binding of Clone 7-STNFRI to TNF.

Assay

1) Coat plate with 2μg/ml of Chemicon recombinant hTNF-α. Block plate w/ 2% BSA.
2) Preincubate Clone 7 periplasm (neat, 1/2, or 1/4) or the purified eukaryotic protein (fraction 3 - pgs 87-89) with ID3 B1 mAb (at 8 or 16 μg/ml) or H398 (at 0.5 or 1 μg/ml) for 30 min. in a 37°C H_2O bath. ↳ or irrelevant N418 Ab at 2μg/ml. Add to the plate
3) Detect the captured STNFRI with 2μg/ml goat α hSTNFRI-(B) Ab.
4) PNPP Incubation = 15 min.

Ig G2a Neutralizing * dilutions of periplasm made in buffer P, spiked with the appropriate concentration of the appropriate Ab.

↳ * The concentration of periplasm or antibody was held constant while the opposite variable changed.

Sample Definitions:

(1) TNF + preincubated periplasm or fraction 3 + goat α hSTNFRI-(B) Ab.

(2) TNF with ID3B1, H398, or N418 alone (no periplasm) + goat α hSTNFRI-(B) Ab.

(3) TNF + periplasm or fraction 3 NOT preincubated with any Ab + goat α hSTNFRI-(B) Ab.

(4) TNF + goat α hSTNFRI-(B) Ab (no periplasm, preincubated or otherwise). * Received 0.1% BSA/PBS/Sweden vitals.

* 4B-E would have been a duplicate of 1B-E.

	(STNFR)	neutralizing Ab (H398)			(ID3 B1)			irrelevant Ab N418	
		0	0.5	1	0	8	16	2	
	↓	1	2	3	4	5	6	7	8
periplasm	A	(4)	(2)	(2)	(4)	(2)	(2)	(2)	
neat	B	(3)	(1)	(1)	(2)	(1)	(1)	(1)	
1/2	C	(3)	(1)	(1)	(2)	(1)	(1)	(1)	
1/4	D	(3)	(1)	(1)	(2)	(1)	(1)	(1)	
fraction 3 neat	E	(3)	(1)	(1)	(2)	(1)	(1)	(1)	

Raw Data Report
Dual Wavelength

	1	2	3	4	5	6	7
A	0.119	0.109	0.112	0.108	0.105	0.094	0.105
B	0.951	0.668	0.510	0.107	0.875	0.788	0.360
C	0.401	0.331	0.301	0.108	0.399	0.373	0.208
D	0.199	0.194	0.178	0.109	0.219	0.216	0.154
E	*.***	*.***	*.***	0.095	*.***	2.369	1.249

* Should have diluted fraction 3 - previous ELISA didn't list the dilution (pg 97), but previous estimates were ~1 µg/ml.

Absorbance Report
Dual Wavelength

Blank
Mean. 0.119
Std.Dev. 0.000

	1	2	3	4	5	6	7
A	0.000	-0.010	-0.007	-0.011	-0.014	-0.025	-0.014
B	0.832	0.549	0.391	-0.012	0.756	0.669	0.241
C	0.282	0.212	0.182	-0.011	0.280	0.254	0.089
D	0.080	0.075	0.059	-0.010	0.100	0.097	0.035
E	*.***	*.***	*.***	-0.024	*.***	2.250	1.130

Summary of Data:

Periplasm	Starting A405 (no Ab)	+ #398 (0.5)	+ #398 (1)	+ ID3 B1 (8)	+ ID3 B1 (16)	+ N418 (2)
neat	0.832	0.549	0.391	0.756	0.669	0.241
1/2	0.282	0.212	0.182	0.280	0.254	0.089
1/4	0.080	0.075	0.059	0.100	0.097	0.035

* There appears to be inhibition of clone 7-derived STNFR I' made to TNF by #398, but not much by ID3 B1 which we previously thought was neutralizing. The irrelevent Ab control, N418, however, may be non-specifically reacting with the periplasm.

10/10/99

10/10 Characterization of Clone 7-derived [illegible] to α-TNF - Inhibition by α-TNF Ab.

In addition to the assay done on 10/4 (pg 100) showing a neutralizing α-STNFRI Ab inhibited the binding of Clone 7 STNFRI to TNF, we want to demonstrate that antibodies to TNF inhibit the binding of Clone 7 STNFRI to TNF. In conjunction, these data support the contention that the Clone 7 STNFRI is folded properly and, therefore, will bind to the active site of ~~[illegible]~~ TNF.

Assays

I. Roxo A, B, & C
- Coat with Chemicon TNF at 2 μg/ml - Block in 2% BSA
- Add α-TNF Ab at 0, 1, 2, or 4 μg/ml (mouse (monoclonal) anti-human TNF-α; Biosource #HC3012 clone B-C7)
- Add STNFR (either: the Clone 7 version purified on the strep-tag column on 10/1 (see pg 98) (these were produced on 9/10) → used fraction 3 diluted 1:2 in Buffer P; or the eukaryotic version made by HeLa cells and purified on the TNF-Affinity Column - used fraction 4 = 254 ng/ml (which was diluted to 20 ng/ml).
- Add goat α hSTNFRI-(B) at 2 μg/ml
- Add KPL SA-AP (at 1:1000)
- pNPP incubation ≅ 10 min.

II Roxo ~~A~~ E
- Coat w/ TNF at 2 μg/ml - Block
- Add the strep-tag, prokaryotic STNFRI (1/2 as described above, fraction 3)
- Add α-TNF Ab at 0, 1, 2, or 4 μg/ml
- Add goat α mouse Ig G, A, & M - (AP) (KPL) at 2 μg/ml
- pNPP ≅ 5 min.

blank=0.189

0.834
blank= 0.519

k=0.519

a

Blank = 0.184

	0	α-TNF Ab (μg/mL) 1	2	4
	1	2	3	4
A	α-STNFR alone (4)	(3)	(3)	(3)
B	(2)	(1)	(1)	(1)
C	(2)	(1)	(1)	(1)
D	right α [illegible] (4)	(3)	(3)	(3)
E	(2)	(1)	(1)	(1)

Key

(1) all Reagents

(2) TNF, STNFR, ND α-TNF Ab, 2° Ab

(3) TNF, ND STNFR, α-TNF Ab, 2° Ab

(4) TNF, ND STNFR, ND α-TNF Ab, 2° Ab

∴ for Assay 1, B1 and C1 are the max signals, with increasing α-TNF Ab, this signal should drop. (B2, B3, + B4 and C2, C3, & C4 respectively)

∴ for Assay 2, D2, D3, and D4 represent maximum binding. A reduction in these signals when our STNFR1 (clone 7) is added suggests that it binds to the active site of TNF and prevents the α-TNF Ab from binding

↳ even though this assay is off-scale, there is a reduction in α-TNF Ab binding

Conclusion

The clone 7-derived hSTNFR1 is properly folded and biologically active.